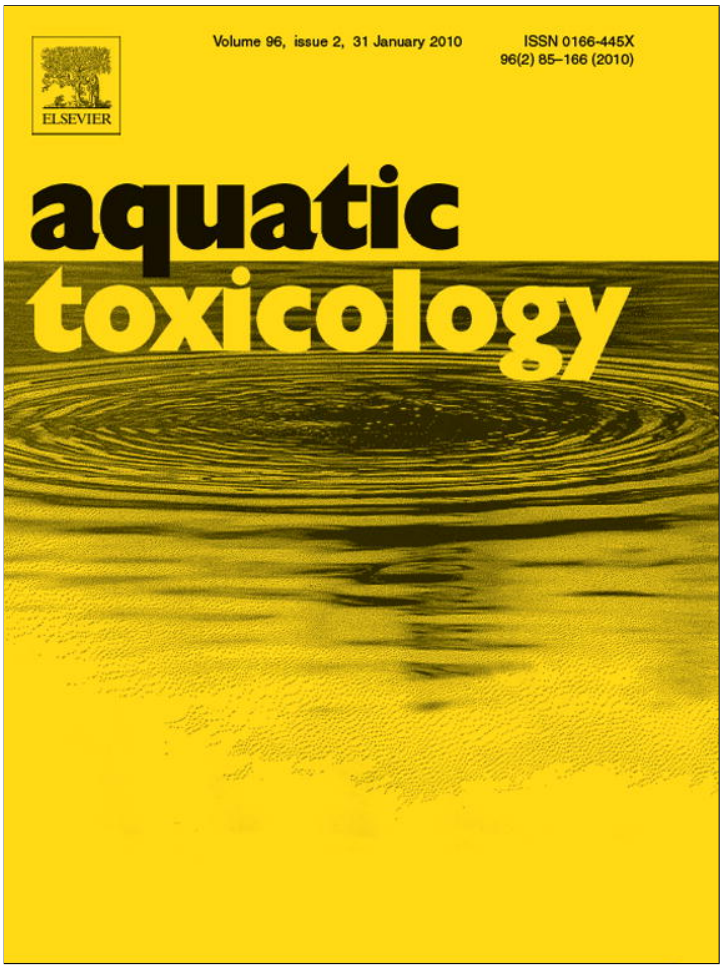

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Effect of dissolved organic matter (DOM) of contrasting origins on Cu and Pb speciation and toxicity to *Paracentrotus lividus* larvae

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ABSTRACT

Water samples of contrasting origin, including natural seawater, two sediment elutriates and sewage-influenced seawater, were collected and obtained to examine the effect of the dissolved organic matter (DOM) present on metal bioavailability. The carbon content (DOC) and the optical properties (absorbance and fluorescence) of the coloured DOM fraction (CDOM) of these materials were determined. Cu and Pb complexation properties were measured by anodic stripping voltammetry (ASV) and the effect of DOM on Cu and Pb bioavailability was studied by means of the *Paracentrotus lividus* embryo-larval bioassay. Sediment elutriates and sewage-influenced water (1) were enriched 1.4–1.7 times in DOC; (2) absorbed and reemitted more light; and (3) presented higher Cu complexation capacities (L_{Cu}) than the natural seawater used for their preparation. L_{Cu} varied from 0.08 μM in natural seawater to 0.3 and 0.5 μM in sediment elutriates and sewage-influenced water, respectively. Differences in DOC, CDOM and Cu complexation capacities were reflected in Cu toxicity. DOM enriched samples presented a Cu EC_{50} of 0.64 μM, significantly higher than the Cu EC_{50} of natural and artificial seawater, which was 0.38 μM. The protecting effect of DOM on Cu toxicity greatly disappeared when the samples were irradiated with high intensity UV-light. Cu toxicity could be successfully predicted considering ASV-labile Cu concentrations in the samples. Pb complexation by DOM was only detected in the DOM-enriched samples and caused little effect on Pb EC_{50}. This effect was contrary for both elutriates: one elutriate reduced Pb toxicity in comparison with the control artificial seawater, while the other increased it. UV irradiation of the samples caused a marked increase in Pb toxicity, which correlated with the remaining DOC concentration. DOM parameters were related to Cu speciation and toxicity: good correlations were found between DOC and Cu EC_{50}, while L_{Cu} correlated better with the fluorescence of marine humic substances. The present results stress the importance of characterizing not only the amount but also the quality of seawater DOM to better predict ecological effects from total metal concentration data.

1. Introduction

Dissolved organic matter (DOM) is considered to decrease metal bioavailability in aquatic environments by binding metal ions and thus reducing the free ion concentration, in accordance with the free ion activity model (FIAM) (reviewed by Campbell, 1995). Laboratory studies using commercial fulvic and humic acids, algal exudates or DOM pre-concentrated by reverse osmosis have confirmed that the FIAM works for both freshwater and seawater organisms exposed to Cu (e.g. Brooks et al., 2007a; Kim et al., 1999; Lorenzo et al., 2002, 2003, 2006; Ma et al., 1999; Wang et al., 2002). Contrasting results have been observed for Pb, the next metal ion with higher affinity for DOM, depending on the DOM type used and the organism tested. Results range from a lower than expected decrease in Pb bioavailability (Lamelas et al., 2005; Lamelas and Slaveykova, 2007; Slaveykova et al., 2003) to an increased bioavailability in the presence of DOM (Sánchez-Marín et al., 2007; Schwartz et al., 2004; Tsiridis et al., 2006). The observed contradiction with FIAM, a model based only on the chemistry of the external medium, has been attributed to an additional effect caused by the direct interaction of DOM with the cell membrane (Campbell et al., 1997; Vigneault et al., 2000; Galvez et al., 2008).

While the above mentioned studies provide useful information on the effects of different DOM components on metal bioavailability, environmental implications cannot be derived without some uncertainty regarding the processes relevant in natural systems. Even the properties of pre-concentrated DOM, such as condensation or coagulation, may change during the reverse osmosis process

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(Maurice et al., 2002) with possible effects on its metal-binding capacity. Furthermore, the applicability of laboratory studies to the field is even more doubtful in the case of seawater, given that the commercially available DOM is isolated from soil or freshwater rather than marine sources.

Based on the current knowledge about the effects of DOM and other ligands on metal speciation and bioavailability, the biotic ligand model (BLM) (Di Toro et al., 2001) has been widely used to predict metal toxicity on the basis of the chemical composition of the medium, the conditional stability constants of metal–ligand interactions (including the "biotic" ligand), and competition with other cations. The concentration of DOC is used in the BLM as a proxy to the amount of DOM in the medium. The U.S. EPA in its ambient freshwater quality criteria for Cu (U.S. EPA, 2007) and recent works for the development of site-specific saltwater quality criteria (Arnold, 2005; Rosen et al., 2005) successfully include the DOC concentrations to fix BLM-based or site-specific-based criteria. Some authors have suggested including other DOM parameters that, in addition to DOC, may improve the predictions of aqueous metal toxicity (e.g. Playle, 1998; Ryan et al., 2004).

Dissolved free and combined amino acids, and humic substances are among the major biogenic coloured DOM (CDOM) compounds that act as metal ligands in the marine environment. They contain chromophores able to reemit the absorbed light as fluorescent radiation (FDOM) in such a way that the FDOM intensity at the specific excitation/emission wavelengths of the protein- and humic-like fluorophores can be used as a proxy to their respective abundance in the medium (Coble, 1996; Nieto-Cid et al., 2006; Yamashita and Tanoue, 2003). Simplicity, sensitivity, quickness and the small sample volume needed are clear advantages of this technique for studying the different contributions of CDOM to the metal–ligand pool in natural waters (Lorenzo et al., 2007).

In the present work, the differences in Cu and Pb complexation properties of seawater samples enriched with DOM of contrasting origins (natural seawater, effluent of a sewage treatment plant, and two sediment elutriates) were studied by anodic stripping voltammetry, and the effect of metal complexation by DOM on Cu and Pb toxicity was evaluated using the *P. lividus* embryo-larval bioassay. Both differences in complexation and in toxicity observed among samples were related to the bulk DOC concentration and the optical properties of the CDOM fraction.

Among the analytical techniques available to determine the ambient chemical speciation of dissolved metals, electrochemical methods, as anodic stripping voltammetry (ASV), are frequently used because of its intrinsic capability in separating ionic metal species from inert complexes. Therefore, they give an estimate of the potential metal detoxification capacity of the water sample, i.e., complexation capacity (Florence, 1986). A fundamental problem underlying efforts to characterize metal complexation in the presence of natural DOM is the heterogeneity of the ligands. DOM heterogeneity can cause several phenomena in ASV measurements, such as the adsorption of DOM on the electrode, the lability or semi-lability of the metal–DOM complexes, and the difference in diffusion coefficient between the free metal and the complexes (e.g. Buffle et al., 1987; Buffle, 1990; Mota and Correia dos Santos, 1995). Despite these interferences, which could induce some underestimation of complexation (Sánchez-Marín et al., 2007), ASV has been proved to be a useful tool to determine Cu bioavailability in seawater (Lorenzo et al., 2002, 2003, 2006; Brooks et al., 2007c) and it has been equally useful for its application to Pb and Zn speciation measurements (Kozelka and Bruland, 1998; Cobelo-García and Prego, 2004).

Sea urchin embryo-larval bioassays have been widely used to assess toxicity in seawater and marine sediments (reviewed by Kobayashi, 1995; Volpi Ghirardini et al., 2005). Sea urchin embryos are, together with bivalve embryos, among the most sensitive organisms to Cu pollution in seawater (U.S. EPA, 2003).

For a complete evaluation of the effects of DOM on metal toxicity, comparison of toxicity in the presence and absence of DOM should be done while keeping constant other characteristics of the medium that could influence metal bioavailability. In the case of natural samples, high intensity UV irradiation of the samples is an effective way to eliminate the organic matter with minimum sample manipulation.

The objectives of the present work are (1) to evaluate the effect of environmentally occurring marine DOM on the speciation and bioavailability of Cu and Pb, oriented to confirm or deny previous laboratory results with commercial DOM, including the ability of ASV-labile metal measurements to predict bioavailability; and (2) to investigate whether DOC concentrations alone are sufficient to describe metal speciation and toxicity in the marine environment or the optical properties of CDOM (absorbance, fluorescence) are also relevant.

2. Materials and methods

2.1. Environmental sample collection and treatment

Four samples of different origins were used in this study: seawater (SW), two sediment elutriates (Elut-1 and Elut-2) and water from an effluent of a sewage treatment plant mixed with seawater (STP). Both sediment resuspension and sewage discharges contribute to the DOM and CDOM pools in coastal waters. Artificial seawater (ASW) prepared according to Lorenzo et al. (2002) was included as a control.

Seawater and sediment samples were collected in the Ría of Vigo, a large coastal inlet in NW Spain, on board of R/V Arao. Seawater was collected at stn P1, in the middle segment of the ría (42°14′58″N–8°46′95″W) with a 5-l Niskin bottle. Subtidal sediment was collected with a Van Veen grab at stns P1 and P2, situated in the middle (42°14′58″N–8°46′95″W) and inner part of the ría (42°17′35″N–8°38′08″W), respectively. Sewage water was collected directly from the effluent of an urban sewage treatment plant from a medium-size town (Cangas, Galicia), whose outlet spills directly into the ría.

Sediment elutriates from stns P1 and P2 were obtained by mixing eight subsamples of 100 g of sediment with 500 ml of SW in air-tight containers with no head space during 30 min, using rotatory agitation as proposed by Beiras (2002). The supernatant was collected after overnight settling at 20 °C. For the STP sample, 400 ml of wastewater were mixed with 4.2 l of SW, to obtain a final salinity of 33.5‰. As our intention was to obtain in laboratory a mixture of wastewater with seawater with a high DOM content, the dilution of wastewater was the minimum possible to ensure an adequate salinity for sea urchin embryos development.

Elutriation is a frequently used method for the evaluation of sediment toxicity as well as for the evaluation of contaminant remobilization from the sediment to the water column (Beiras, 2002; USEPA, 2001). It reproduces in laboratory the worst-case scenario of sediment resuspension that can occur in coasts due to storms, dredging operations, etc. Wastewater effluents dumping directly to the coast, both treated and untreated, are a source of contaminants as well as organic matter to the surrounding waters. Both sediment resuspension and sewage discharges contribute to the DOM and CDOM pools in coastal waters.

All samples except ASW, which is free of particles, were filtered through 0.45 μm polyethersulfone filters and the filtrate was collected and pooled in a four litre polycarbonate bottle. In order to oxidize the organic matter, one half of each sample was UV-digested for 2 h using a UV-Digestor equipped with a high-pressure

mercury lamp of 200 W similar to the one described in Achterberg and Van den Berg (1994).

Both untreated and UV-treated samples (2 l each) were subsampled for subsequent measurements and experiments: 125 ml were collected in glass bottles for fluorescence, absorbance and DOC measurements, and the rest was divided in plastic bottles and kept frozen for later measurements of dissolved metal concentrations, complexation capacity and the toxicity bioassays.

All plastic-ware was washed with diluted bleach, rinsed and soaked in 5% nitric acid for 24 h and rinsed several times with ultrapure water before use. Glassware was combusted at 500 °C for 4 h to eliminate the organic matter. All sample manipulations were performed in a laminar flow cabinet using clean laboratory practices for metals.

2.2. DOM characterization

2.2.1. Dissolved organic carbon (DOC)

DOC samples were collected into 10 ml precombusted (450 °C, 12 h) glass ampoules. After acidification with H_3PO_4 to pH < 2, the ampoules were heat-sealed and stored in the dark at 4 °C until analysis. DOC was measured with a Shimadzu TOC V-CPH organic carbon analyser (Álvarez-Salgado and Miller, 1998). The system was standardized daily with potassium hydrogen phthalate. The concentration of DOC was determined by subtracting the instrument blank area from the average peak area and dividing by the slope of the standard curve. The CV was ~1% and the accuracy of the measurements was successfully tested with the blank (<1 μM) and Sargasso Sea deep water (44.0 ± 1.5 μM) TOC reference materials provided by Prof. D. Hansell (University of Miami).

2.2.2. Fluorescence of dissolved organic matter

Fluorescence was measured using a Perkin–Elmer LS 55 Luminescence Spectrometer, equipped with a xenon lamp (equivalent to 20 kW for 8 μs duration) and a 1 cm quartz fluorescence cell. Milli-Q water was used as a blank and the intensity of the Raman peak was checked regularly (Nieto-Cid et al., 2005).

Discrete excitation/emission pair measurements were performed at 320 nm/410 nm, characteristic of the marine humic-like substances (FDOMm), and 280 nm/350 nm, characteristic of the protein-like substances (FDOMt) (Coble et al., 1990; Nieto-Cid et al., 2005). Four replicate measurements were performed for each Ex/Em wavelength. A five-point standard curve was prepared daily with a mixed standard of quinine sulphate (QS) and tryptophan (Trp) in sulphuric acid 0.05 M (Nieto-Cid et al., 2005). The equivalent concentration of every peak was determined by subtracting the blank height from the average peak height, and dividing by the slope of the standard curve. FDOMm was expressed in ppb equivalents of QS (ppb QS) and FDOMt in ppb equivalents of Trp (ppb Trp).

2.2.3. Absorbance of dissolved organic matter

The absorbance of the samples between 250 and 500 nm was measured using a Beckman spectrophotometer, equipped with a 10 cm quartz cell. Milli-Q water was used as blank. The absorption coefficient at 350 nm ($aDOM_{350}$) has been used in this study as a proxy to the concentration of CDOM in the samples. It is calculated multiplying the absorbance at 350 nm times 2.303 and divided by the length of the cuvette in metres.

2.3. Copper and lead complexation

The complexation of copper or lead by DOM was studied using voltammetric titrations with the metal under study. The mercury drop electrode used in ASV detects only a fraction of the dissolved metal (the labile metal), which comprises the free ion plus the inorganic metal complexes. Because inorganic composition is practically constant in seawater, the free metal ion is a constant fraction of the labile metal, and therefore labile metal measurements can be used to test the FIAM.

Water samples were thawed, shaken and dispensed in several (from 9 to 13) 20 ml polystyrene vials. Increasing additions of Cu (or Pb) were made to the vials, to obtain metal concentrations ranging from 0.025 to 2 μM. Cu and Pb were obtained from standard solutions of 1 g l^{-1} in the form $Cu(NO_3)_2$ and $Pb(NO_3)_2$ in 0.5N HNO_3 (Panreac Química SA; Barcelona, Spain). Solutions were kept 24 h in the dark to allow equilibration of the complexation reaction. Copper and lead titrations were made separately. It was checked that the metal additions did not alter the pH.

Electrochemically labile metal was measured by square wave anodic stripping voltammetry (ASV). Analyses were carried out with a hanging mercury drop electrode, a Ag/AgCl reference and a Pt-rod auxiliary electrode held in a Metrohm 663 VA polarographic stand coupled to an Eco-Chemie AutoLab PGSTAT10 potentiostat. Measurements were performed in a Teflon cell thermostatted at 20 °C. The cell was pre-conditioned with 10 ml of each sample for 5 min, then the solution was discarded and the remaining 10 ml of sample were measured. Solutions were purged for 250 s with N_2. Copper was accumulated on a mercury drop of 0.52 mm^2 at −0.5 V for 20 s at the maximum stirring speed (3000 rpm) and 10 s of equilibration were allowed before the voltage scan. Lead was accumulated during 5 s at −0.65 V and 5 s of equilibration time were allowed. The conditions of the squarewave (SW) scan were an initial potential equal to the deposition potential, a SW amplitude of 25 mV, a SW frequency of 25 Hz and a scan increment of 2 mV. Three voltammograms were recorded for each solution, and the arithmetic mean of peak intensities was transformed into labile metal concentrations ([Cu′] or [Pb′]) dividing by the slope of the titration curve after the natural organic ligands have become saturated with metal (Donat et al., 1994). The titration curves were explained assuming the simplest complexation model; that is, only one type of ligand and a reaction stoichiometry of 1:1. Titration plots were fitted to Eq. (1), obtained from the theoretical complexation model previously explained (Lorenzo et al., 2002).

$$M' = \frac{-a + \sqrt{a^2 + 4M_T/K'}}{2} \quad (1)$$

where $a = (-M_T + L + 1/K')$, and M_T and M' are the total and labile metal concentrations (mol l^{-1}), respectively. L is the total ligand concentration (mol l^{-1}) and K' is the conditional stability constant of the M–L complexes, valid for the conditions of the experimental medium. M represents Cu or Pb, depending on the complexation curve.

Total dissolved metal concentrations were also measured in the filtered and UV-digested samples. For that, an aliquot of each sample was acidified to pH 2 with trace metal grade HNO_3 (Scharlau Chemie S.A) and UV-digested as previously explained. Zn, Cd, Pb and Cu concentrations were measured by standard additions by ASV using a deposition potential of −1.1 V and 120 s of deposition time. The parameters of the squarewave scan were the same described for the complexation measurements.

2.4. Sea urchin embryo-larval bioassay

Adult sea urchins were collected from a subtidal population of the Ría de Vigo (NW Spain), immediately transported to the laboratory in a cool box, kept in a 200 l aquarium at 12–18 °C, and fed with *Ulva lactuca* and boiled mussels.

Experimental solutions were prepared in 50 ml polypropylene vials, in which the different samples (defrosted and vigorously shaken) were dispensed and five metal additions of Cu (or Pb) were made. Metal exposure concentrations were: 0, 0.2, 0.5, 0.75, 1 and

Table 1
Concentration of dissolved organic carbon (DOC, μM), fluorescent pseudo-protein materials and other aromatic compounds (FDOMt, ppb Trp), fluorescent humic substances (FDOMm, ppb QS) and coloured dissolved organic matter ($aDOM_{350}$, m^{-1})[a].

	DOC	FDOMt	FDOMm	$aDOM_{350}$
ASW	11.5 ± 2.1	3.1 ± 0.1	1.2 ± 0.1	0.23 ± 0.21
SW	100.7 ± 1.2	15.0 ± 0.3	5.2 ± 0.1	0.46 ± 0.16
Elut-1	155.4 ± 1.3	40.5 ± 0.3	23.0 ± 0.3	7.99 ± 1.80
Elut-2	170.5 ± 0.5	98.5 ± 0.5	23.9 ± 0.3	4.72 ± 2.99
STP	146.0 ± 1.3	119.9 ± 0.5	50.3 ± 0.3	1.75 ± 0.23
ASW-UV[b]	9.2 ± 0.2	1.0 ± 0.1	0.3 ± 0.1	0.16 ± 0.16
SW-UV[b]	40.8 ± 0.8	1.4 ± 0.1	0.6 ± 0.1	0.09 ± 0.16
Elut-1 UV[b]	42.2 ± 2.0	2.7 ± 0.1	0.7 ± 0.1	1.52 ± 0.23
Elut-2 UV[b]	44.8 ± 0.8	0.4 ± 0.1	0.4 ± 0.1	0.69 ± 0.23
STP–UV[b]	56.2 ± 2.2	1.4 ± 0.1	0.4 ± 0.1	0.35 ± 0.23

[a] Mean ± standard deviation of the measurement is given.
[b] UV after the name of the sample indicates a UV-radiated sample.

2 μM for Cu and 0, 0.2, 0.5, 1, 2 and 5 μM for Pb. These concentrations were chosen based on previous knowledge of the toxicity curves (Lorenzo et al., 2002; Sánchez-Marín et al., 2007). Two extra-treatments (0.1 μM-Cu and 10 μM-Pb) were included for the ASW. After metal additions, solutions were shaken and left to equilibrate for 24 h at 20 °C in dark conditions. Physicochemical parameters (pH, dissolved oxygen and salinity) were measured using standard electrodes (Metrohm 744 pH Meter, a YSI 5000 probe for O_2, and a conductivity cell Tetracon 325 for salinity). These measurements were performed in an aliquot of the samples with no metal addition (C_0 samples) prior to the inoculation of gametes. This aliquot was only used with that purpose and was discarded after the measurement. An aliquot of 20 ml of each treatment was taken and frozen for posterior checking of Cu′ and Pb′ concentrations by ASV.

Sea urchin bioassay was performed following the procedures of Fernández and Beiras (2001). After the fertilization, around 80 eggs (20 μl) were delivered into 4 ml polypropylene vials containing the test solutions to get a final density of 20 eggs per ml. The vials (four replicates per treatment) were incubated at 20 °C for 48 h in the dark to avoid photodegradation of the DOM. After the incubation period, larvae were fixed with a few drops of 40% formalin. For each individual replicate, the length of 35 individuals was recorded under inverted microscope as the endpoint of the bioassay.

The percentage of larval growth (*%LG*) was calculated for each replicate assuming that the 100% of larval growth was the mean larval growth in the corresponding C_0 sample.

Data were fitted to the following log-logistic model:

$$\%LG = \frac{100}{1 + \left([M]/EC_{50}\right)^{a}} \quad (2)$$

where [M] is the concentration of metal, *a* is the Hill slope of the toxicity curve and EC_{50} is the median effective concentration.

2.5. Data treatment and statistics

Correlations between variables were studied using the Pearson correlation coefficient (*r*) calculated with the statistical software SPSS 15.0 for Windows (SPSS Inc.). Two different correlation analyses were performed, one grouping all data and another considering only the non-digested data. The first one is highly influenced by the absence of organic matter in the UV-digested samples and, therefore, positive correlations will stress the effect of the absence/presence of DOM. The second one is more suitable to detect correlations between variables in the samples with DOM from contrasting origin.

Non-linear fittings were adjusted by least squares using SigmaPlot 2002 (8.0) for Windows (SPSS Inc.). Statistical differences between variables' values from different samples were tested using a standard *t*-test or *t*-test with Welch correction. The later was used when variances of the compared values were unequal (Sokal and Rohlf, 1995). Homogeneity of variances was tested using a standard *F*-test. The comparison of EC_{50} was done by the extra sum-of-squares *F*-test by means of global fitting (Motulsky and Christopoulos, 2003), using GraphPad Prism version 4.00 for Windows (GraphPad software).

Table 2
Matrix of Pearson correlation coefficients (first line) among the variables of DOM characterization, complexation capacity and Cu and Pb EC_{50}, including significance level (second line) and sample size (third line)[a].

	All samples							Non-digested samples						
	FDOMt	FDOMm	$aDOM_{350}$	L_{Cu}	L_{Pb}	Cu EC_{50}	Pb EC_{50}	FDOMt	FDOMm	$aDOM_{350}$	L_{Cu}	L_{Pb}	Cu EC_{50}	Pb EC_{50}
DOC	0.836 0.003 10	0.807 0.005 10	0.762 0.010 10	0.866 0.001 10	0.977 0.137 3	0.839 0.002 10	0.237 0.510 10	0.738 0.155 5	0.685 0.202 5	0.684 0.203 5	0.795 0.108 5	0.977 0.137 3	0.870 0.055 5	0.014 0.983 5
FDOMt		0.949 0.000 10	0.483 0.157 10	0.958 0.000 9	−0.339 0.780 3	0.815 0.004 10	0.278 0.437 10		0.914 0.030 5	0.245 0.691 5	0.932 0.021 5	−0.339 0.780 3	0.771 0.127 5	−0.141 0.821 5
FDOMm			0.508 0.134 10	0.982 0.000 9	−0.891 0.299 3	0.843 0.002 10	0.398 0.254 10			0.286 0.641 5	0.973 0.005 5	−0.891 0.299 3	0.830 0.082 5	0.178 0.775 5
$aDOM_{350}$				0.616 0.077 9	0.547 0.632 3	0.811 0.004 10	0.482 0.158 10				0.469 0.425 5	0.547 0.632 3	0.769 0.128 5	0.568 0.318 5
L_{Cu}					−0.838 0.367 3	0.894 0.001 9	0.390 0.300 9					−0.838 0.367 3	0.924 0.025 5	0.203 0.743 5
L_{Pb}						−0.590 0.598 3	−0.349 0.773 3						−0.590 0.598 3	−0.349 0.773 3
Cu EC_{50}							0.527 0.117 10							0.405 0.499 5

[a] DOC is the dissolved organic carbon concentration; FDOMt and FDOMm represent the protein- and humic-like fluorescence, respectively; $aDOM_{350}$ is the absorption coefficient at 350 nm and L_{Cu} and L_{Pb} are the Cu and Pb complexation capacities of the samples.

Statistical analyses were considered significant at the $p < 0.05$ level unless otherwise noted.

The limit of detection (LOD) of complexation capacity was estimated as $3 \times S_L$, where S_L is the standard error of the lowest complexation capacity detected.

3. Results and discussion

3.1. DOM characterization

The five samples obtained for this study were characterized by different DOC concentrations and optical properties (Table 1). As expected, the ASW sample showed the lowest values for all the measured parameters because it is made from high-quality DOM-free reagents. The SW sample values were within the natural ranges found in the Ría de Vigo (e.g. Doval et al., 1997; Nieto-Cid et al., 2005). On the contrary, the elutriates and the STP sample were enriched with DOM released from either the sediments or the sewage effluent, having higher values than the SW sample for all the studied variables.

The DOC concentrations for Elut-1 and Elut-2 were 1.5 and 1.7 times higher than for SW, respectively. For the STP sample, the DOC enrichment due to the dilution with sewage water was similar, 1.4 times higher than for SW. The original DOC concentration in the outlet of the sewage plant, as estimated from the dilution, was about 620 μM.

Regarding the fluorescence of CDOM, the STP sample presented the highest values, for both humic- and protein-like materials (50.3 ppb QS and 119.9 ppb Trp, respectively). Elutriates showed similar values of FDOMm (23.0 and 23.9 ppb QS for Elut-1 and Elut-2, respectively) but Elut-2 displayed a higher FDOMt (98.5 ppb Trp), closer to the STP value. On the other hand, absorption coefficient was higher in Elut-1 (7.99 m^{-1}), while the value for the STP sample was unusually low (1.75 m^{-1}) in contrast with its high fluorescence.

Due to the marked decrease in DOC, CDOM and FDOM levels caused by UV irradiation, strong correlations were found between DOC and the three optical parameters (Table 2) when all samples (digested and non-digested) are considered in the correlation analysis. When just the untreated samples were considered, a significant correlation was observed only between FDOMm and FDOMt (Table 2). Although FDOMm and DOM absorbance are both used to characterize CDOM (Coble, 2007), these two parameters did not correlate in this study. Elutriates presented the lowest FDOMm/$aDOM_{350}$ ratios (2.9 ± 0.5 and 5 ± 3 ppb QS m, respectively, for Elut-1 and Elut-2), while the ratio for the STP sample was 10 times higher than for Elut-1 (29 ± 4 ppb QS m). This difference could be attributed to molecular size of DOM (Midorikawa and Tanoue, 1998 and references therein), suggesting that humic substances in the STP sample are predominantly of low molecular weight, whereas humic substances released from the sediments presented higher molecular weights.

The two elutriates displayed different DOM characteristics: Elut-1 presented higher $aDOM_{350}$, while DOC and FDOMt were higher for Elut-2, and the two samples presented very similar values for FDOMm. These differences can be attributed to the different composition of the organic matter present in the sediments. Unpublished data from a pollution assessment of the Ría de Vigo revealed that the percentage of organic carbon in sediments from P1 was 2.3%, while in P2 it was 4.0%. This difference of 1.7 times was not displayed by the DOC values of the elutriates, while it was well reflected by the FDOMt. This fact suggests that the higher organic content of sediments from P2 was composed mainly by protein-like and other aromatic compounds, and that the composition of the organic matter from P2 might include a high percentage of compounds which are not easily remobilized to the dissolved phase.

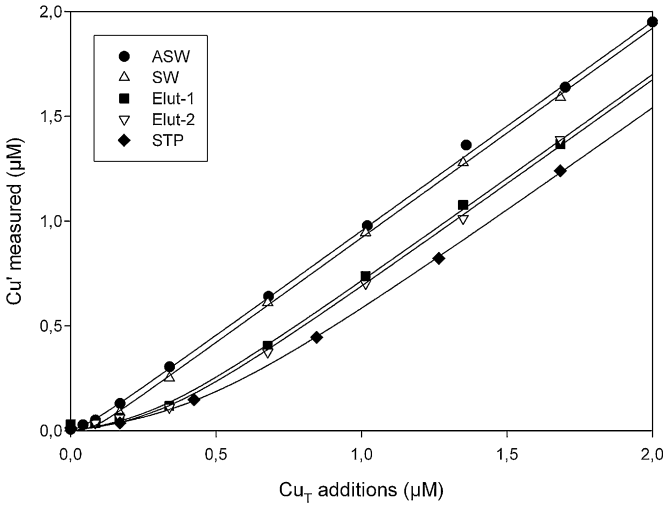

Fig. 1. Cu titrations of the non-digested samples. Solid lines represent the best non-linear squares fittings of the individual curves to Eq. (1).

After UV irradiation (Table 1), the bulk DOC decreased from 60% to 75%, whereas the decrease in fluorescence and absorbance ranged from 85% to 99% of CDOM. These data indicated that the samples contained a higher percentage of materials resistant to UV radiation in the bulk DOC than in the CDOM fraction. Values of FDOMm after UV degradation are very low reflecting the high photo-reactivity of humic substances.

3.2. Cu and Pb complexation

The complexation model by Lorenzo et al. (2002) successfully explained the titration data (r^2 ranged between 0.995 and 0.999). Fig. 1 shows the fittings to the Cu-titrations of non-digested samples. Samples with higher complexation capacity need a larger amount of Cu to achieve ligand saturation and, therefore, they present curves shifted to the right. Complexation parameters (L and log K') are shown in Table 3. In some samples, Pb complexation capacity was too low to be detected by the methodology used. The range of metal ligands which can be detected depends on the analytical window of the technique (Apte et al., 1988) as well as, for this particular technique (ASV), on the parameters chosen for the measurement (especially deposition time) and the metal concentrations added for the titration. Our methodology was chosen to detect changes in [M′] in the range of *P. lividus* embryo sensitivity. Therefore, samples with a lower ligand concentration will not be detected by our experimental approach. We have calculated a lower limit of detection of L for the methodology used, which is 0.027 μM for Cu and 0.01 μM for Pb. Both values are far below the toxicity thresholds observed for *P. lividus* larvae (Fernández and Beiras, 2001).

Copper ligand concentrations in untreated samples were low for ASW and SW, and higher for both elutriates and STP. As for the DOM parameters, elutriates and STP samples are enriched with ligands released from sediments or from the effluent water. For SW, the value is within the range reported by other authors in estuaries and rías (e.g. Santos-Echeandia et al., 2008a; Cobelo-García and Prego, 2004; Donat et al., 1994). Regarding the sediment elutriates, Cobelo-García and Prego (2004) have also showed that a resuspension of the sediment involved an increase in ligand levels of the overlying waters. Undiluted sewage water had a Cu complexation capacity of 4.8 μM, as calculated from the ligand concentration in the STP sample. This value is within the range reported by

Table 3
Parameters (±standard error) obtained from the fitting of the ASV measurements to the complexation model (Eq. (1)) for Cu and Pb[a].

	L_{Cu}	log $K_{Cu'}$	r^2 (n)	L_{Pb}	log $K_{Pb'}$	r^2 (n)
ASW	0.048 ± 0.012**	7.41 ± 0.84***	0.999 (10)	–	–	–
SW	0.080 ± 0.007**	8.06 ± 0.63***	0.999 (8)	–	–	–
Elut-1	0.313 ± 0.018***	7.14 ± 0.19***	0.999 (8)	0.202 ± 0.035***	6.12 ± 0.21***	0.999 (11)
Elut-2	0.339 ± 0.019***	7.18 ± 0.19***	0.999 (8)	0.322 ± 0.104*	5.83 ± 0.3***	0.999 (11)
STP	0.491 ± 0.013***	6.97 ± 0.07***	0.999 (6)	0.042 ± 0.003***	7.46 ± 0.26***	0.999 (11)
ASW-UV	0.039 ± 0.006**	7.75 ± 0.41***	0.999 (6)	–	–	–
SW-UV	0.049 ± 0.012**	8.42 ± 1.74*	0.995 (5)	–	–	–
Elut-1 UV	0.041 ± 0.004**	8.66 ± 0.71**	0.999 (5)	–	–	–
Elut-2 UV	0.027 ± 0.009*	7.72 ± 1.05***	0.998 (9)	–	–	–
STP–UV	0.037 ± 0.020 ns	6.86 ± 0.96***	0.998 (8)	–	–	–

Empty values represent samples whose complexation capacity was too low to be detected. LOD of L was calculated to be 0.027 μM (Cu) and 0.01 μM (Pb).
Signification of parameters is marked by ns (no significant), *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$).
[a] L is the complexation capacity of the water in μM of metal; log K' is the logarithm of the conditional stability constant of the complexes; the coefficient of determination (r^2) and the number of additions used for each complexation curve (n) are also reported.

Santos-Echeandia et al. (2008b) for several sewage waters in the area.

Apparent conditional stability constants (log K') represent an average value of the stability constants of all the ligands present in each sample. Logarithm of conditional stability constants varied between 6.97 and 8.06, with the highest value found in SW and the lowest one in STP. However, the precision of calculated log K' values is not sufficient to differentiate between samples. Only the log K' from STP differs from the SW value at $p = 0.094$ (Welch's t-test), suggesting that the majority of the ligands released from the sewage treatment plant bind copper more weakly than the natural ligands present in SW.

After UV-digestion, a pronounced decay of ligand concentration occurs in all samples (between 40 and 90%, Table 3) indicating that most of the complexing ligands were broken down during the UV-treatment.

In the case of Pb, only the STP and the elutriates presented detectable ligand concentrations (>0.01 μM) given the deposition time and the metal loading range used. After UV-treatment, no Pb complexing ligands were detected in any sample, showing that they were destroyed during the UV oxidation of DOM.

Pb was less affected than Cu by DOM complexation. Conditional stability constants in elutriates are one order of magnitude lower for Pb than for Cu, and only the samples with a considerably higher L_{Cu} (both elutriates and STP) show some affinity for Pb. It is remarkable that the sample with the highest L_{Cu} (STP), shows in comparison very little L_{Pb}, suggesting a different binding behaviour of these two metals to the ligands present in this water.

Fitted complexation curves were used to calculate predicted labile Cu and Pb in the bioassay solutions from the total metal concentrations (initial plus added metal). Comparison of predictions with measurements of labile metal performed in the samples was done for both metals. Good linear relationships of measured vs predicted values were obtained, with slopes of 1.04 ± 0.02 ($r^2 = 0.96$; df = 46) for Cu′ and 0.996 ± 0.009 ($r^2 = 0.99$; df = 49) for Pb′.

3.3. Cu and Pb toxicity to P. lividus larvae

3.3.1. Physicochemical parameters and larval growth in C_0 samples

Different larval growth was achieved in the C_0 samples (Table 4). The highest larval growth was observed in ASW and SW, and it did not change after digestion of these samples (t-test). Mean larval growth for these four samples was 374 ± 16 μm. Concerning non-digested samples, a lower larval growth (from 40 to 50% reduction) is observed in both elutriates and in the STP sample.

Measured pH, salinity and dissolved oxygen in C_0 samples (represented in Table 4) were within the limits reported for optimal growth of *P. lividus* larvae (Saco-Alvarez et al., in press). Total dissolved metal concentrations, measured in the samples prior to any metal addition, were also lower than reported LOECs for *P. lividus* larvae (Fernández and Beiras, 2001). Maximum metal concentrations were observed in the STP sample, and they were 0.017 μM for Cu, 0.04 μM for Pb, 0.16 μM for Zn and 0.3 nM for Cd. Metal concentrations were not altered by the UV-digestion process.

Therefore, reduction in larval growth in the elutriates and STP without metal addition cannot be attributed to any of the measured physicochemical characteristics of the samples, and could be due to the presence of other contaminants in the sediments and the sewage effluent which have not been measured in the present study. The sediments of the two sites chosen for this study have been exhaustively characterized in a previous work, and P1 (site of Elut-1) is considered a reference site with low levels of pollution, while P2, moderately contaminated, presented levels of PAHs and PCBs in the sediments lower than the threshold effects level in sediment quality guidelines (Macdonald et al., 1996).

A decrease in larval growth has been systematically observed in elutriates from unpolluted sites (Fernández, 2002; Saco-Álvarez, 2008), going from 5 to 20% of decrease with respect to larval growth in filtered seawater. This effect is enhanced in finer and more organically enriched sediments, and it has been related to the

Table 4
Parameters (S‰, pH and DO-dissolved oxygen) measured in the samples prior to the inoculation of gametes, mean larval growth (LG) ± std ($n = 4$) observed in the samples with no added metal and Cu and Pb EC_{50} calculated as a function of total metal and labile metal.

Sample	Salinity (‰)	pH	DO (mg/L)	LG (μm)	Cu EC_{50} (μM Cu_T)	Cu′ EC_{50} (μM Cu′)	Pb EC_{50} (μM Pb_T)	Pb′ EC_{50} (μM Pb′)
ASW	34.9	8.08	7.02	384 ± 19	0.35 ± 0.01	0.31 ± 0.01	1.96 ± 0.08	1.96 ± 0.08
ASW-UV	34.6	8.15	7.12	370 ± 15	0.356 ± 0.005	0.316 ± 0.004	2.03 ± 0.06	2.03 ± 0.06
SW	35.4	8.12	7.19	378 ± 14	0.38 ± 0.01	0.29 ± 0.01	1.64 ± 0.10	1.64 ± 0.10
SW-UV	35.8	8.08	7.13	363 ± 13	0.253 ± 0.004	0.201 ± 0.004	1.72 ± 0.09	1.72 ± 0.09
Elut-1	35.1	7.84	7.14	186 ± 12	0.66 ± 0.03	0.39 ± 0.02	2.32 ± 0.12	2.17 ± 0.12
Elut-1 UV	35.6	7.94	7.25	261 ± 16	0.45 ± 0.01	0.41 ± 0.01	1.56 ± 0.08	1.56 ± 0.08
Elut 2	35.1	7.98	7.27	203 ± 5	0.60 ± 0.02	0.31 ± 0.02	1.64 ± 0.08	1.47 ± 0.07
Elut-2 UV	31.0	7.91	7.37	219 ± 16	0.37 ± 0.01	0.34 ± 0.01	1.33 ± 0.10	1.33 ± 0.10
STP	33.1	8.12	7.23	219 ± 16	0.64 ± 0.02	0.28 ± 0.01	1.93 ± 0.17	1.87 ± 0.17
STP–UV	31.6	8.03	7.32	335 ± 13	0.27 ± 0.01	0.27 ± 0.01	0.99 ± 0.05	0.99 ± 0.05

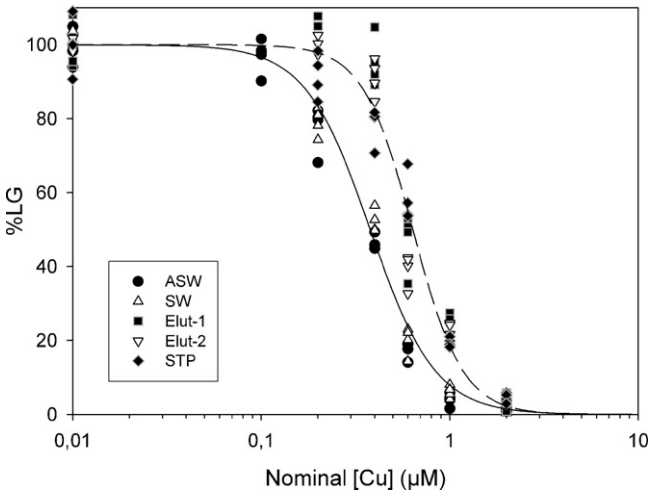

Fig. 2. Results of the bioassay and fitted Cu toxicity curves for non-digested samples. Solid line represents the common fitting for ASW and SW data (EC_{50} = 0.38 and a = 2.52) and dashed line represents the common fitting for the other three samples (EC_{50} = 0.64 and a = 3.28).

physicochemical characteristics of the sediments in the absence of chemical contamination (Long et al., 1990).

The lower larval growth observed in the elutriates and the STP is partially recovered after UV-digestion of the samples, which could indicate that UV-digestion partially destroys the agent that causes this effect.

For the sake of simplicity, it was assumed in first instance that the observed different larval growth in the C_0 samples does not have any influence on Cu and Pb toxicity. The effect of DOM on metal toxicity will be assessed independently of other characteristics of the sample that could influence larval growth, regardless of metal concentration.

3.3.2. Cu toxicity curves and relation to Cu speciation

Fig. 2 presents the fittings of the toxicity model (Eq. (2)) to the Cu toxicity data. Nominal total Cu concentrations correspond to added Cu plus the Cu originally present in each sample. Fittings successfully explained from 96 to 99% of the variability of data, and fitted parameters (EC_{50} and a) were significant for all curves. According to the extra sum-of-squares F-test, ASW and SW data were pooled together and fitted to a single curve, while data from the other three samples (both elutriates and STP) were also pooled and fitted to another curve. The EC_{50} of the first group (SW-ASW) was 0.38 μM of Cu, while the elutriates-STP showed a higher EC_{50} (0.64 μM). Therefore both elutriates and STP had a protecting effect against Cu toxicity, presumably due to the Cu complexation by DOM. In contrast, the DOM present in SW is not sufficient to reduce Cu toxicity significantly as compared to ASW. Fitted EC_{50} for each individual curve are shown in Table 4 and represented in Fig. 3a. After the UV-treatment, EC_{50} decreases in all samples except in ASW. The fact that EC_{50} remains unaltered for ASW proves that the UV-treatment itself does not have an influence on Cu toxicity, and therefore the higher toxicity observed after the UV exposure of the other samples might be caused by the UV-destruction of DOM. The protecting effect of DOM on Cu toxicity, which gave rise to Cu EC_{50} values of 0.64 μM for the elutriates and STP, disappeared or was greatly reduced after the UV oxidation of DOM, resulting in a decrease in EC_{50} down to 0.27–0.45 μM of Cu. Possible presence of oxygen radicals and H_2O_2 in the UV-irradiated samples is ruled out from the interpretation given that these compounds might have disappeared from the samples during the freezing–thawn process or during the 24 h of metal equilibration left until the bioassay was performed.

In order to better understand whether differences in Cu toxicity are due to Cu complexation capacity of the samples, labile Cu concentrations were calculated from the total Cu concentrations and the complexation parameters, applying Eq. (1). The results of fitting the toxicity data to Eq. (2) using [Cu′] instead of $[Cu]_T$ are presented in Table 4, and Cu′ EC_{50} values are depicted in Fig. 3b. Comparing Fig. 3a to Fig. 3b it is clear that [Cu′] explains toxicity much better than $[Cu]_T$, since the difference between digested and undigested samples largely disappears. For the three DOM-enriched samples, the differences in Cu EC_{50} before and after UV-treatment-observed in Fig. 3a-totally disappear when speciation is considered (Fig. 3b), and therefore these differences can be attributed solely to Cu complexation by DOM. However, for SW, the Cu′ EC_{50} after the UV-treatment is still lower than for the non-digested sample, and therefore the higher toxicity of the digested sample could not be attributed to the oxidation of DOM. Ideally, according to FIAM, toxicity expressed in [Cu′] should be the same for all samples and equal to the toxicity in ASW, i.e. there should be no differences in the Cu′-EC_{50} values. As can be observed in Fig. 4a, this is true for all samples except for the already mentioned SW–UV and for Elut-1. The latter showed slightly lower toxicity compared to the other samples, both before and after the UV-digestion.

Despite those discrepancies, in general, the behaviour of Cu agrees with that expected on the basis of FIAM and the ASV measurements, as shown in Fig. 4a, where predicted and observed Cu EC_{50} values are compared. Predictions were made considering that 0.31 μM of Cu′ (observed value in ASW) will cause 50% of reduction of larval growth regardless of the sample (given that variations in salinity and pH are too low to have an influence). Then the $[Cu]_T$ that will give a [Cu′] of 0.31 was calculated based on the complexation parameters obtained by the voltammetric titrations. Toxicity in non-digested samples very accurately adjusted to predictions, while larger variations were observed in some of the UV-digested samples. Furthermore, significant correlations were observed between Cu EC_{50} and L_{Cu} considering both all and non-digested samples (Table 2). An 85% of the observed variability of Cu toxicity could be explained by L_{Cu}.

The present results generally agree with the bioavailability models and confirm for seawater and seawater with added DOM of contrasting environmental origins that Cu bioavailability is a function of the free Cu concentration. Other studies have also found accurate relationships of Cu bioavailability with Cu speciation in the presence of natural DOM in freshwater (Sunda and Lewis, 1978; Luider et al., 2004; Mylon et al., 2003) and seawater (Rivera-Duarte et al., 2005).

3.3.3. Pb toxicity curves and relation to Pb speciation

Fittings of the toxicity curve to the observed percentage of larval growth successfully explained from 96 to 99% of data variability and fitted parameters (EC_{50} and a) were significant for all curves.

In contrast to Cu, Pb toxicity was less affected by the organic matter present in the samples, and the observed effects were different (protecting, enhancing or not affecting toxicity) depending on the sample. Fig. 3c and Table 4 show the Pb EC_{50} values. Pb EC_{50} in ASW was 1.96 μM, and a similar value was obtained in the UV-treated ASW, showing that the UV-treatment itself does not have any influence on Pb toxicity. Only Elut-1 seemed to exert a protecting effect on Pb toxicity compared to control water (ASW). In contrast, in SW and Elut-2, Pb toxicity was increased, while STP did not change Pb EC_{50} in comparison with ASW.

After the UV-treatment, Pb toxicity remains unaltered for ASW and SW, while it increases for the three DOM-enriched samples, even when no protecting effect has been observed in these samples before UV-exposure.

When speciation was considered and EC_{50} values were calculated considering [Pb′] (Table 4 and Fig. 3d), some differences in

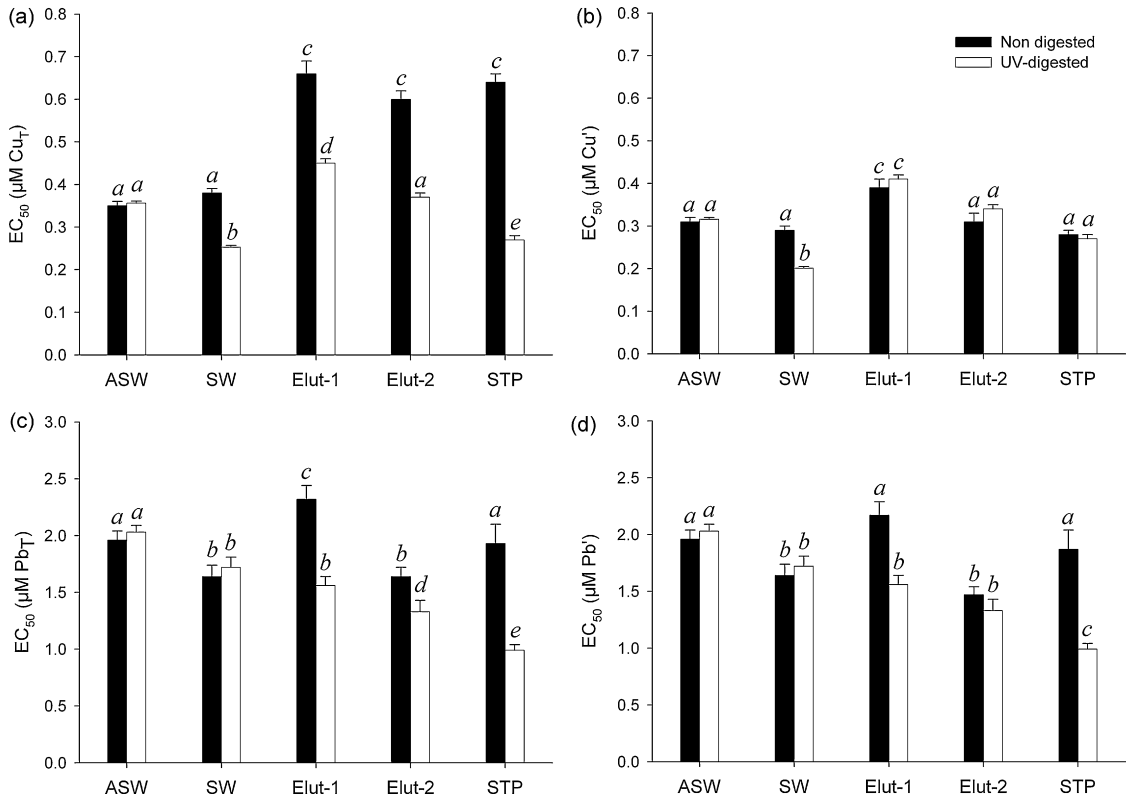

Fig. 3. Observed Cu EC_{50} (a) and (b) and Pb EC_{50} (c) and (d) in samples before and after UV destruction of organic matter calculated considering total (initial + added) metal concentration in solution (a) and (c) and labile metal calculated from complexation parameters (b) and (d). Significant differences between EC_{50}s at the $p = 0.05$ level are marked by letters (from a to e), each letter representing a group of non-different EC_{50}.

Pb toxicity were explained: the protecting effect of Elut-1 was explained by Pb complexation by DOM given that the obtained EC_{50} in the basis of Pb′ – 2.17 (± 0.12) μM of Pb′ – is not significantly different from the 1.96 (±0.08) μM value of ASW; also the differences in Pb toxicity observed between Elut-2 and Elut-2 UV are not significant when speciation is considered. But still the decrease in EC_{50} after UV-digestion for Elut-1 and STP cannot be explained considering Pb speciation only.

It is not surprising that there is almost no protecting effect of the DOM on Pb toxicity given that Pb complexation capacity (L_{Pb}; Table 1) is lower than that of Cu, especially in comparison with their relative EC_{50} values (the ratio L/EC_{50} is much lower for Pb). Pb EC_{50} is 5.6 times higher than Cu EC_{50}, therefore it would need also a five fold increase in complexation for a similar protecting effect to be observed. Based on the complexation parameters, only the elutriates were supposed to have a protecting effect on Pb toxicity on the basis of FIAM, but this effect would increase EC_{50} only by 10% compared to the treatment without DOM. Therefore, predicted EC_{50} values do not present a high variation (Fig. 4b). Observed values for the non-digested samples are within 10% variation from pre-

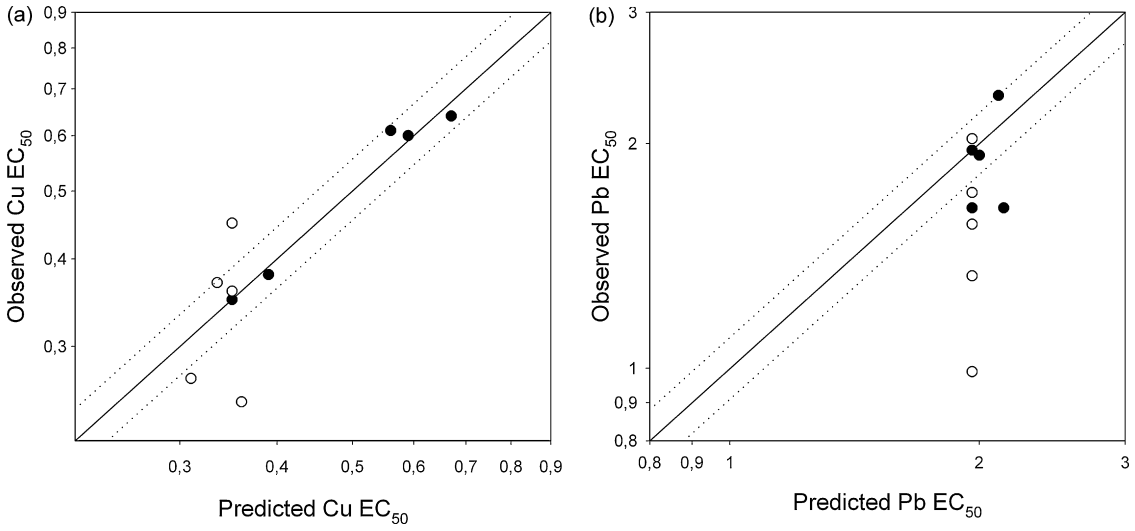

Fig. 4. Observed vs predicted EC_{50} for (a) Cu and (b) Pb expressed in μM. Black dots represent non-digested samples and open dots represent UV-treated samples. Prediction is based on the complexation curves obtained by ASV assuming that EC_{50} based on [M′] are 0.31 μM of Cu and 1.96 μM of Pb. Solid line represents the 1:1 line prediction–observation and pointed lines represent the ± 10% deviation from the 1:1 line.

diction except for SW and Elut-2, which show higher toxicity than expected. Even though humic acid has been shown to increase Pb toxicity to *P. lividus* larvae (Sánchez-Marín et al., 2007), in this case the extra-toxicity observed in SW and Elut-2 cannot be attributed to the presence of DOM because it is not reduced after the UV-treatment (Fig. 3a, white bars). Furthermore, as observed in Fig. 4b (white dots) some of the UV-digested samples depart clearly from prediction. In both elutriates and STP, there is a decrease in EC_{50} with the UV-digestion, reaching the lowest value of 0.99 μM of Pb in the STP–UV sample, which is half of the value observed in ASW. It seems that during the UV-digestion, some component is formed in those waters, which results in toxicity to *P. lividus* larvae in the presence of Pb.

The results presented here showed that Pb toxicity is more difficult to model as a function of speciation than Cu toxicity. Cu complexation with DOM resulted in all cases in a protecting effect against Cu toxicity. In contrast, the two samples presenting considerable Pb complexation capacity showed opposite effects. One elutriate showed a protecting effect against Pb toxicity while in the other Pb toxicity was higher than in artificial seawater. Although there are no other studies about Pb bioavailability in the presence of DOM in seawater, similar results have been observed in freshwater studies. Schwartz et al. (2004) observed opposite effects of DOM on Pb toxicity depending on DOM source, some decreasing and others increasing Pb toxicity as compared to Pb-only exposures. Lamelas et al. (2005) demonstrated that equal DOC concentrations of different compositions had a different effect on Pb bioavailability for a freshwater microalgae, being some of them adequately predicted according to free ion concentrations and others not.

Evaluation of the effect of DOM on Pb toxicity in seawater is limited by the lower toxicity of Pb in comparison with Cu. In order to cause toxicity it is thus necessary to use Pb concentrations orders of magnitude above background levels in the sea. Sea urchin embryos are among the most sensitive organisms to acute metal exposure, and a more sensitive response to Pb pollution can only be obtained by resorting to chronic exposure tests. Long term bioavailability studies are complicated, since speciation in exposure solution should be controlled and kept constant over the whole exposure period.

3.4. Relation of Cu and Pb complexation and bioavailability with DOM properties

3.4.1. Relation of complexation capacity with DOM characterization

Correlations between DOM parameters (DOC, FDOMt, FDOMm and $aDOM_{350}$) and Cu and Pb complexation capacities (L_{Cu} and L_{Pb}) are summarised in Table 2 and graphically represented in Fig. 5. When all data are considered, L_{Cu} correlates significantly ($p < 0.05$) with all DOM parameters except $aDOM_{350}$. As previously discussed, these correlations are strongly influenced by the UV-digestion process, which highly reduces fluorescence, DOC, and L_M values. Correlation analysis using non-digested samples only will be more suitable to elucidate which DOM parameters are better descriptors of metal complexation capacity. When only undigested samples are considered the correlation between L_{Cu} and DOC is no longer significant. The parameter that better explains L_{Cu} is the fluorescence of humic-like substances, that explains 95% of the Cu complexation capacity ($p = 0.005$). This relationship is described by the equation: $L_{Cu} = 0.06\,(\pm 0.03) + 0.009\,(\pm 0.001)$ FDOMm ($r^2 = 0.95$; $n = 5$). This fact indicates that fluorescence is a better descriptor of Cu–ligands present in natural waters than DOC concentrations. This observation has been noted before during a simulated bloom of a marine diatom by Lorenzo et al. (2007). These authors observed a DOC decrease that was followed by an increase in Cu complexation capacity. Fluorescence of CDOM was able to explain the lack of correlation between DOC and L_{Cu}. Apte et al. (2000) also observed with freshwater samples that Cu complexation capacity correlated better with fluorescence of humic substances than with DOC concentrations.

Regarding Pb, only three samples are available for the correlation analysis (given that complexation capacity was too low in the other seven samples), and no significant correlation was found between L_{Pb} and any of the studied DOM properties. Despite the lack of significance at the $p < 0.05$ level, it can be observed from the Pearson correlation coefficient that 95% of L_{Pb} can be explained by DOC. This fact, in contrast with the lack of correlation between L_{Cu} and DOC for the same samples, suggests a different binding behaviour of Cu and Pb with DOM, as has been previously shown for humic substances (Christl et al., 2001).

Other studies performed with freshwater have also related DOM optical properties with Cu complexation characteristics (Haitzer et al., 1999; Brooks et al., 2007b,c). These studies have found good correlations between Cu–DOM stability constants or L_{Cu} normalized by DOC and DOM aromaticity, which is estimated from specific absorbance coefficients (normalized to DOC) or measured by NMR. A significant correlation between L_{Cu} normalized to DOC and other DOM variable means that DOC is not enough to completely explain variations in ligand concentrations, otherwise variations in normalized values would be insignificant. Because not all carbon present in the DOM pool contributes equally to Cu complexation, DOC was insufficient to explain differences in complexation and better correlations were observed when a DOM quality parameter accounting for the differences in complexation per gram of DOC was included. Using FDOM to predict Cu complexation capacity is a similar approach, in the sense that it combines in one single variable both the quantity and quality of DOM. FDOM indirectly estimates the amount of fluorescent functional groups, which are also considered metal-binding functional groups, as have been demonstrated by the use of fluorescence quenching in metal complexation studies (Ryan and Weber, 1982). However, very few studies have investigated the potential use of FDOM as predictor of metal complexation capacity in natural waters (Apte et al., 2000; Lorenzo et al., 2007).

3.4.2. Relation of Cu and Pb toxicity with DOM parameters

The correlation of metal toxicity with the different DOM parameters is also summarised in Table 2. Cu toxicity correlates with all DOM parameters when all data are considered. These correlations highlight the protecting effect of the presence of DOM on Cu toxicity. Samples with higher DOM content (higher DOC, FDOM and $aDOM_{350}$) needed more dissolved Cu to exert the same toxicity as less Cu caused in samples with low DOM. The second analysis, considering only the non-digested samples, can be used to detect which of the measured DOM parameters describe better the different degrees of protection of the different samples. None of the studied parameters correlate with Cu EC_{50} at the $p < 0.05$ level, although both DOC and FDOMm correlate with Cu EC_{50} with more than a 90% of confidence ($p < 0.10$). DOC is the parameter that better explains Cu EC_{50}, slightly better than FDOMm. Looking at the correlations in detail (Fig. 5), it can be observed that for low DOC concentrations (<100 μM), DOC is not a good predictor of Cu toxicity as samples with very different DOC (SW and ASW) presented a similar Cu EC_{50}. In contrast, FDOMm shows larger variability for DOM-enriched samples (Elutriates and STP), while EC_{50} is very similar for those three samples. The best prediction of EC_{50} was achieved with the multiple regression equation: Cu $EC_{50} = 0.268\,(\pm 0.018) + 0.007\,(\pm 0.001)$ FDOMm + 114 (±17) $aDOM_{350}$/DOC ($r^2 = 0.960$; $p < 0.001$; $n = 10$). The second variable ($aDOM_{350}$/DOC) is the specific absorption coefficient (SAC) and has been used by other authors as a DOM quality factor to correct for differences in metal complexation capacity per unit DOC between DOM sources (see discussion below).

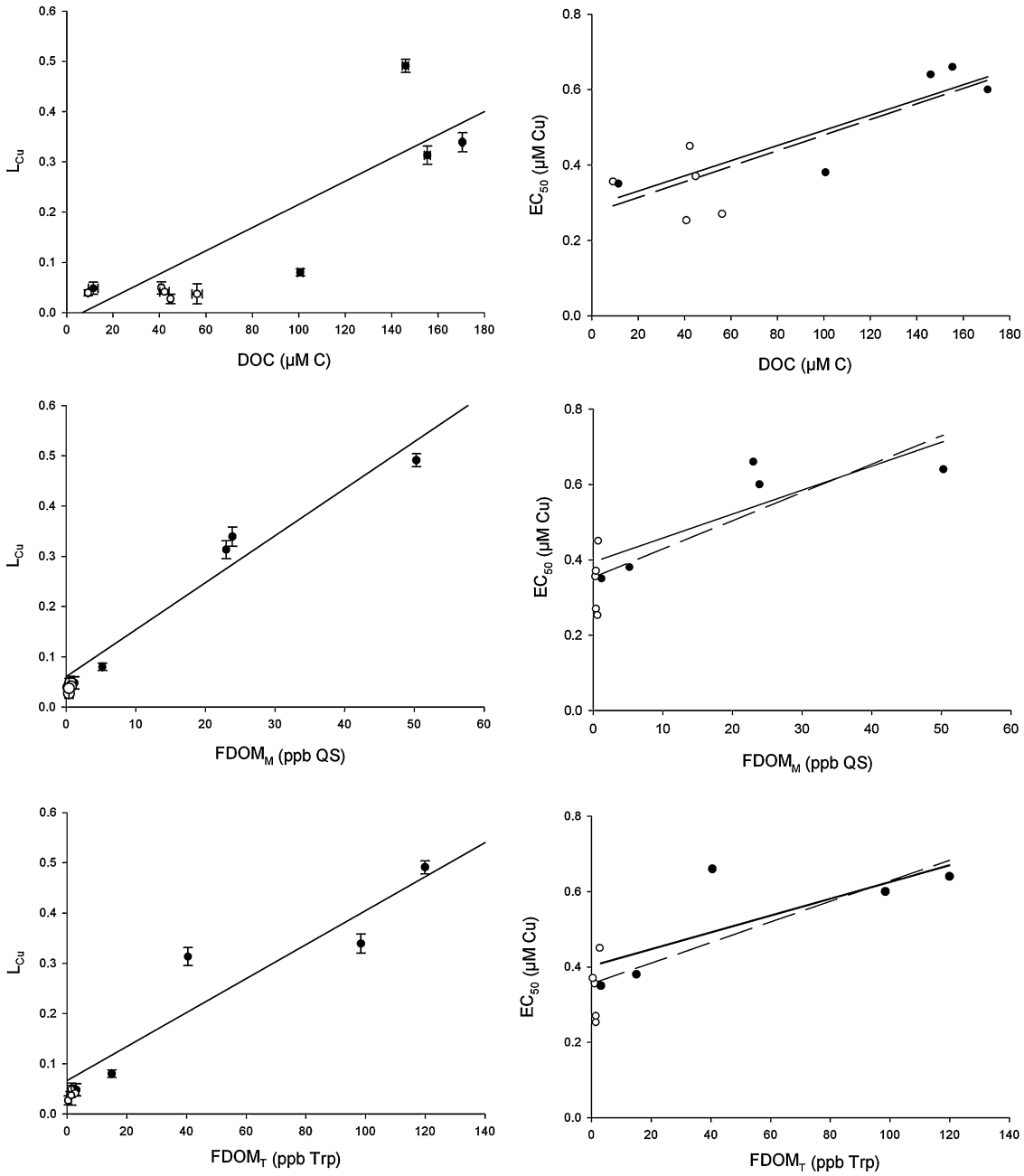

Fig. 5. Correlations between Cu complexation capacity and DOC, FDOMt and FDOMm (left side) and correlations of Cu EC_{50} with L_{Cu}, DOC and FDOMm (right side). Black dots represent the non-digested samples and open dots the UV-treated ones. Error bars (±*st dv*) are represented when larger than symbol size. Solid lines represent the correlations with non-digested samples only (black dots) and dashed lines represent the correlations including digested samples (white dots) also.

Regarding Pb, no correlation was found between Pb EC_{50} values and the DOM parameters, either when all data or non-digested data were considered. As explained in Section 3.3.3, this lack of correlation can be due to the low ratio L_{Pb}/EC_{50}, i.e. the effect of DOM on Pb speciation is not sufficient to greatly influence Pb toxicity for *P. lividus* given the relatively high EC_{50} for Pb (1.96 μM) as compared to Cu (0.31 μM).

One significant correlation was found for the UV-digested samples, between Pb EC_{50} and [DOC] ($r = -0.903$, $p = 0.036$, $n = 5$), but this correlation was negative (Fig. 6). This observation, contrary to the expected protecting effect of DOM, is in concordance with the high Pb toxicity observed after the UV-digestion of some samples and which was attributed to an unknown component formed in the waters. This DOC might be low molecular weight organic matter resulting from the breaking down of more complex DOM compounds. There is evidence of the production of low molecular weight carbonyl compounds in a wide variety of natural waters upon irradiation with sunlight (Kieber et al., 1990). It could be possible that the UV-digestion broke the large DOM molecules into smaller compounds, which could be able to transport Pb into the organisms (e.g. piggy-back transport).

In an extensive compilation of data from 21 sites in different USA bays, Arnold (2005) found a significant relationship between DOC and Cu EC_{50} for *Mytilus* sp. larvae. Following the relationship obtained, EC_{50} values could be predicted within a factor of ±2, which means that observed EC_{50} values can fall somewhere between the double and the half of predicted values, a remarkable precision considering the variable composition of the natural samples used. In the light of the present work, even better predictions are likely to be achieved if some measurement of Cu

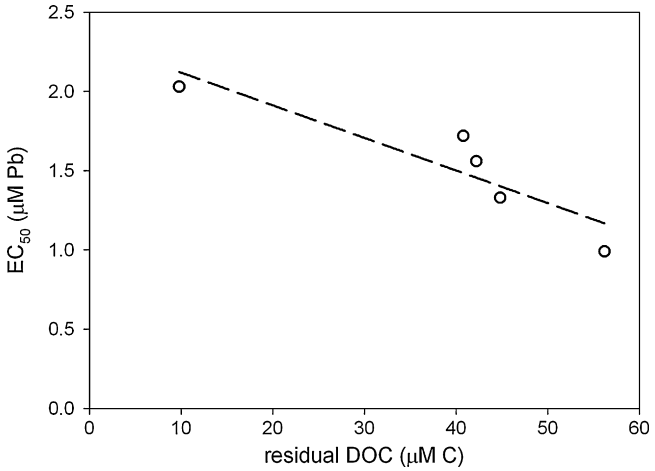

Fig. 6. Pb EC_{50} in UV-digested samples related to residual [DOC] remaining in the samples after UV-digestion.

speciation or further DOM characterization would be included. A similar study has also shown significant correlations between EC_{50} and DOC (Rosen et al., 2005) in coastal waters. Very few reports have included the study of optical properties of DOM for the evaluation of metal toxicity in seawater. Only Pempkowiak et al. (1999) made a first attempt to relate spectroscopic and chemical properties of DOM to metal bioavailability in seawater, using DOM extracted from different freshwater sources. They found that 60% of the differences in Cu accumulation by mussels at constant DOM concentrations were explained by the carbon content in organic matter, while DOM aromaticity (expressed as a ratio of specific absorbances at 250 and 270 nm) explained 13% of the variability. Nadella et al. (2009) used fluorescence excitation–emission matrix spectroscopy to characterize DOM from different freshwater sources, and reported higher protecting effect at higher fulvic to humic ratio, but did not include any quantitative relationship between DOM quality and metal toxicity.

In freshwater, more work has been done including DOM quality measurements for the improvement of bioavailability models. Different organic matter sources have been shown to decrease Cu toxicity or uptake by fish gills to a variable extent (Richards et al., 2001; Luider et al., 2004; Ryan et al., 2004; Schwartz et al., 2004; De Schamphelaere et al., 2004). All these studies postulate that autochthonous DOM, with lower aromaticity, bound less metal per unit DOC than allochthonous DOM. The authors propose the inclusion of an optical measurement (specific absorption coefficient; SAC) as a parameter to derive DOM quality factors to include in bioavailability models, and recommend the inclusion of fluorescence measurements for future research. Similar work was also performed for Pb (Richards et al., 2001; Macdonald et al., 2002; Schwartz et al., 2004), showing the ability of SAC to explain differences observed in Pb toxicity or Pb uptake by fish gills caused by DOM of different sources.

All these studies stress the need of including DOM quality measurements in bioavailability models or site-specific water quality criteria for Cu. In the present work, with only five samples tested, it is not possible to obtain a good estimate of which DOM parameter will explain Cu toxicity best. Possibly a combination of several DOM properties, as DOC with SAC or FDOM with SAC (which explained 97% of our data variability), will improve toxicity predictions.

4. Conclusions

This work adds more evidence to the extended idea that Cu toxicity in aquatic systems is controlled by Cu ion activity. It has been confirmed with environmental samples of contrasting origin (natural seawater, elutriates and sewage-influenced water) that anodic stripping voltammetry is a useful tool for predicting Cu toxicity in seawater irrespectively of the origin of DOM. In the case of Pb, we have shown that Pb speciation is affected by the DOM present in resuspended sediments, but to a lesser extent than Cu speciation. However, conclusions about how DOM will affect Pb bioavailability, are strongly limited by the low ratio L_{Pb}/EC_{50}, i.e. the relatively high concentration of Pb necessary to cause a 50% of reduction in the growth of *P. lividus* larvae. More work should be done at environmentally relevant Pb concentrations, using Pb bioaccumulation as endpoint. In general, DOM effect on Pb toxicity is more variable than for Cu. It has been observed an increase in Pb toxicity presumably caused by residual DOC remaining in the samples after UV irradiation, not occurring for Cu in the same samples. This observation adds evidence to the fact previously reported that some types of DOM can increase Pb toxicity in seawater.

Our results stress the importance of characterizing the DOM content of seawater in order to predict ecological effects from total metal concentration data. Good correlations were found between DOC and Cu EC_{50}, and fluorescence measurements appear as a promising tool for the prediction of Cu complexation capacity in seawater.

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